

Influence of the *Escherichia coli* Capsule on Complement Fixation and on Phagocytosis and Killing by Human Phagocytes

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ABSTRACT To define mechanisms by which polysaccharide capsules confer enhanced virulence on gram-negative bacteria, we examined the effect of the *Escherichia coli* capsule on complement fixation to the bacterial surface and on phagocytosis and killing of these bacteria by mouse macrophages and human polymorphonuclear leukocytes (PMN) and monocytes. When *E. coli* were attached to mouse macrophages with concanavalin A, the macrophages readily phagocytosed unencapsulated but not encapsulated bacteria even in the presence of fresh mouse serum; macrophages did not phagocytose encapsulated *E. coli* unless antibacterial or anti-Con A antibody was added. Similarly, when these bacteria were attached to human PMN with Con A, PMN ingested unencapsulated but not encapsulated *E. coli*.

PMN phagocytosed and killed encapsulated serum-resistant *E. coli* only in the presence of both complement and antibacterial antibody; PMN phagocytosed and killed unencapsulated *E. coli* of the same strain in the presence of complement alone. Fluorescence microscopy showed that antibody had to be present for encapsulated but not unencapsulated *E. coli* to fix complement to its surface.

To examine the role of the complement receptors of human PMN and monocytes in phagocytosis and killing of encapsulated *E. coli*, we used human and rabbit antibacterial immunoglobulin (Ig)M to fix complement to the bacteria. PMN and monocytes phagocytosed and killed encapsulated *E. coli* in the presence of both IgM and complement, but not in the presence of either serum opsonin alone. In the presence of antibacterial

IgG, PMN and monocytes required complement to effectively phagocytose and kill the *E. coli*.

We conclude that (a) attachment by itself results in ingestion of unencapsulated but not encapsulated *E. coli*; (b) under physiologic conditions, *E. coli* are not phagocytosed or killed effectively by PMN or monocytes unless complement is fixed to their surface; (c) in the absence of antibody, the *E. coli* capsule blocks complement fixation to the bacterial surface probably by masking surface components, such as lipopolysaccharide, capable of activating the complement pathway; (d) the *E. coli* capsule imposes a requirement for specific antibacterial antibody for complement fixation; and (e) the complement receptor of human PMN and monocytes mediates phagocytosis of complement-coated encapsulated bacteria and is the primary mediator of phagocytosis and killing of these bacteria.

INTRODUCTION

For many bacterial species, the presence of a capsule has been identified as a critical determinant of virulence. For some, such as *Escherichia coli*, the degree of virulence is a function of both the size and composition of the capsule (1). In experimental animals, encapsulated forms are more virulent than unencapsulated forms (2, 3), and the degree of virulence is proportional to the quantity of capsular (K) antigen expressed on the bacterial surface (4).

Most *E. coli* isolated from human infections are encapsulated (1, 5). *E. coli* isolated from the urine of patients with pyelonephritis contain especially large amounts of capsular antigen (6–8), and certain capsular serotypes, most notably K1, predominate in these patients and in infants with *E. coli* meningitis (6, 9–11).

In view of the importance of encapsulated *E. coli* and other encapsulated gram-negative bacteria as human pathogens, it is surprising that so little is known of the role(s) of the capsule in enhancing virulence. Several investigators have reported that a capsule re-

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duces the bactericidal effects of serum on *E. coli* and enhances the ability of this organism to resist phagocytosis (2, 4, 12, 13). Others have shown that anticapsular antibody has a protective effect on rabbits and mice inoculated with encapsulated *E. coli* (14, 15). These findings suggested to us that the capsule may alter the binding of complement to the *E. coli* surface.

Complement activation by *E. coli* and other gram-negative bacteria has been examined almost exclusively in unencapsulated strains. Thus, although it is known that unencapsulated *E. coli* fix complement via the alternative complement pathway (16–18), virtually nothing is known about complement fixation or consumption by encapsulated *E. coli*. Similarly, studies of phagocytosis of *E. coli* have focused on unencapsulated *E. coli*, particularly the laboratory-adapted and rough *E. coli* K12 strain, or on encapsulated *E. coli* that have been heat-killed and thereby have had their capsule destroyed. Such studies may not be relevant to the in vivo situation where pathogenic bacteria are encapsulated.

We undertook this study to define the mechanisms by which capsules promote virulence of gram-negative bacteria. In our studies, we used an encapsulated *E. coli* strain (serotype 09:K29:H[−]) originally isolated from human peritoneum (19), and unencapsulated mutant strains bearing the 09 somatic antigens. We chose these strains because the chemical structures of the 09 somatic (20) and K29 capsular (21) antigens have been determined and both are composed of repeating polysaccharide subunits containing the specific carbohydrate-combining site (22) for the plant lectin concanavalin A (Con A).¹ The presence of Con A binding sites on the bacteria allowed us to attach the bacteria to the surface of mouse and human leukocytes with this lectin and study bacteria-phagocyte interactions in the absence of the two major serum opsonins, antibody and complement. We shall demonstrate that the presence of a capsule markedly alters the interactions of *E. coli* with serum complement and with the surface of phagocytic leukocytes. Additionally, we shall characterize the roles of antibody and complement in phagocytosis and killing of encapsulated *E. coli* by phagocytic leukocytes.

METHODS

Reagents. Con A, three times crystallized and lyophilized (Miles-Yeda Ltd., Kankakee, Ill.); α -methyl-D-mannopyranose

side (α -MM), grade III (Sigma Chemical Co., St. Louis, Mo.); glutaraldehyde 50% (Electron Microscopy Sciences, Fort Washington, Pa.).

Antisera and standards. Goat anti-Con A (Miles-Yeda Ltd.); goat anti-human IgG, anti-human IgM, anti-rabbit IgG, anti-rabbit IgM, all heavy chain specific; goat anti-human complement C3, IgG fraction, rhodamine conjugated; and human fraction rich in IgG (no IgA, no IgM) or IgM (no IgA, no IgG) (N. L. Cappel Laboratories Inc., Cochranville, Pa.).

Media. Dulbecco's phosphate-buffered saline with Ca⁺⁺ and Mg⁺⁺ ions (PBS) (23); Hanks' balanced salt solution (HBSS) 10 × (Grand Island Biological Co., Grand Island, N. Y.), adjusted to pH 7.4; PBS without Ca⁺⁺ and Mg⁺⁺ (PD); tryptic soy broth (TSB) (Difco Laboratories, Detroit, Mich.); minimal essential medium without glucose (24).

Polymorphonuclear leukocytes (PMN). Blood from a single healthy donor (type AB+) was collected in a heparinized syringe, mixed 1:1 with dextran 6% wt/vol in 0.9% saline (Dextran 75, Abbott Laboratories, Chicago, Ill.), and sedimented at 24°C. PMN were separated from the leukocyte-rich supernate by centrifugation over a discontinuous gradient of Ficoll (Ficoll 400, Pharmacia Fine Chemicals, Piscataway, N. J.) and sodium diatrizoate (Hypaque sodium 50%, Winthrop Laboratories, New York) as described (25). The granulocyte-rich fraction thus obtained was washed twice in PBS (4°C) by centrifugation at 200 g for 10 min at 4°C after which residual erythrocytes were lysed by a 1-min exposure to 0.2% saline. Isotonicity was rapidly restored with 1.6% saline, and the cells (>99% PMN) were pelleted, resuspended in HBSS, counted in an improved Neubauer Chamber (Clay Adams, Div. Becton, Dickinson & Co., Parsippany, N. J.), stored at 0°C at the required concentration and used within 30 min of purification. Viability was >99% by the criterion of trypan blue exclusion.

Human monocytes. Heparinized blood was diluted 1:1 in 0.9% saline and the mononuclear fraction obtained by centrifugation at 800 g for 30 min at 24°C over a Ficoll-sodium diatrizoate solution (Ficoll-Paque, Pharmacia Fine Chemicals) as described (26). The mononuclear cells were collected, washed, purified of erythrocytes, and resuspended in HBSS exactly as described for PMN. A sample of the suspension was cytocentrifuged (Cytospin, Shandon Southern Instruments Inc., Sewickley, Pa.) and stained. A differential cell count was done in each experiment, and the monocyte concentration was adjusted to the required concentration; the cell population (>99% viable by trypan blue exclusion) contained ~1.5% PMN, 40% monocytes, and 58.5% lymphocytes.

Mouse peritoneal macrophages. Resident and thioglycollate-elicited mouse peritoneal macrophages were harvested and cultured on 13-mm Diam glass coverslips as described (27).

Bacteria. *E. coli*, serotype 09:K29:H[−] (*E. coli* K⁺), strain Bi 161-42, was kindly provided by Dr. Frits and Dr. Ida Ørskov, International Escherichia Centre, Statens Serum Institut, Copenhagen, Denmark. *E. coli*, serotype 09:K29:H[−] (*E. coli* K[−]), a spontaneously occurring unencapsulated mutant of strain Bi 161-42, was isolated from a colony of the parent strain on the basis of colonial morphology. The mutant was shown to have a biochemical and antibiotic sensitivity (antibiogram) profile identical to the parent strain; these tests were generously performed by the Laboratory of Microbiology, The New York Hospital-Cornell Medical Center, New York. *E. coli*, serotype 09:K9:H12 (*E. coli* 09:K9:H12), strain ×43, an unencapsulated mutant of *E. coli*, serotype 09:K9:H12, strain Bi 316/42, was also provided by Dr. Ørskov.

E. coli K⁺, K[−], and 09:K9:H12, adjusted to 1% vol/vol suspensions in PBS (OD standardized, see below), were agglutinated by ≥ 333 , ≥ 4 , and ≥ 16 μ g/ml of Con A. *E. coli*

¹Abbreviations used in this paper: Abs S, human serum absorbed with *E. coli* K⁺ and K[−]; AD, agglutinating dose; CFU, colony-forming units; Con A, concanavalin A; *E. coli* K⁺, *E. coli*, serotype 09:K29:H[−]; *E. coli* K[−], *E. coli*, serotype 09:K29:H[−]; HBSS, Hanks' balanced salt solution; HI, heat-inactivated; NS, normal human serum; PBS, phosphate-buffered saline with Ca⁺⁺ and Mg⁺⁺ ions; PD, PBS without Ca⁺⁺ and Mg⁺⁺; PMN, human polymorphonuclear leukocytes; RBC, erythrocytes; TSB, tryptic soy broth.

K⁺ was strongly agglutinated (and exhibited a quellung reaction) with rabbit anti-09:K29 antiserum (prepared against the strain); it was not agglutinated by rabbit anti-09 antiserum (prepared against boiled *E. coli*, serotype 09:K9:H12, strain Bi 316/42). The *E. coli* K⁻ and 09:K9⁻:H12 strains were agglutinated by both the anti-09:K29 and the anti-09 antisera. Both antisera were kindly provided by Dr. Ørskov. *E. coli* K⁺ exhibited a large capsule when examined by Preisz' wetfield India Ink method (28).

For stock cultures, a highly mucoid *E. coli* K⁺, an *E. coli* K⁻, or an *E. coli* 09:K9⁻:H12 colony was grown in TSB at 37°C on a rotatory shaker to mid-log phase (OD of 0.300 at 540 nm measured in a Coleman 44 model spectrophotometer [Perkin-Elmer Corp., Norwalk, Conn.]) and the culture stored at -70°C in 10% glycerol. As needed, cultures were thawed and cultured on TSB agar. An appropriate colony was picked, grown up on TSB agar, and held at room temperature for 2–7 d. 14–18 h before an experiment, an appropriate colony was again picked and grown up on TSB agar at 37°C; a standard inoculum from this growth was suspended in 150 ml of TSB and grown at 37°C to an OD of 0.300 at 540 nm. The bacteria were then collected by centrifugation at 1,500 g for 10 min at 4°C, washed twice, and resuspended in PBS (4°C) to the required concentration, stored at 4°C, and used within 1 h; there was no loss in colony-forming units (CFU) on storage for at least 4 h. A 1% suspension of *E. coli* K⁺ adjusted to an OD of 0.410 at 540 nm yielded 2.65×10^8 CFU/ml; 0.2% suspensions of *E. coli* K⁻ and *E. coli* 09:K9⁻:H12 adjusted to an OD of 0.510 at 540 nm yielded 2.5 and 2.8×10^8 CFU/ml, respectively.

Human sera. Venous blood was clotted in siliconized glass test tubes, stored for 1 h at room temperature and an additional 1 h at 4°C, and the normal serum (NS) separated and stored at -70°C. Complement activity was inactivated by heating serum at 56°C for 30 min just before use. Absorbed serum was prepared by successively incubating 30 ml of a 1:1 mixture of NS with PD three times with fresh batches of 10^{11} *E. coli* K⁺ and/or K⁻ on a gyratory shaker at 4°C for 1 h; the bacteria had been grown to mid-log phase, washed in PD, and pelleted by centrifugation. Absorbed serum was separated from the bacteria by centrifugation, filtered (0.45 μ m Millipore filters; Millipore Corp., Bedford, Mass.), and stored at -70°C.

Complement activity: classic pathway. Hemolytic activity for antibody-coated sheep erythrocytes (RBC) in normal and absorbed human serum was measured as described (29). Normal human serum had 365 CH₅₀ U/ml, serum absorbed with *E. coli* K⁺ had 200 CH₅₀ U/ml, serum absorbed with *E. coli* K⁻ had 200 CH₅₀ U/ml, and serum absorbed with both organisms had 250 CH₅₀ U/ml.

Complement activity: alternative pathway. Hemolytic activity of normal and absorbed sera for rabbit RBC was measured by a modification of the method of Platts-Mills and Ishizaka (30). A standard hemolysis curve (percentage RBC on ordinate; OD units on abscissa) was generated by incubating 200 μ l of a suspension of rabbit RBC in concentrations ranging from 0 to 1% vol/vol in EGTA buffer (30) with 400 μ l of water for 5 min at 4°C, adding 2.5 ml of EGTA buffer (4°C), centrifuging, and determining the OD of the supernate at 412 nm on a Coleman 44 spectrophotometer. Sera were assayed by incubating 200 μ l of 1% rabbit RBC with 0–400 μ l of serum in EGTA buffer for 30 min at 37°C, stopping the reaction by adding 2.5 ml of EGTA buffer (4°C), centrifuging, and determining the OD of the supernate. The percentage of hemolysis (*y*) was determined from the standard hemolysis curve, and the 50% hemolytic titer was obtained graphically on log-log paper by plotting on the *x*-axis the amount of serum added, and on the *y*-axis the ratio *y*/100-*y*;

1,000 divided by the amount of serum in microliters giving 50% hemolysis (*x* intercept) yielded CH₅₀ alternative pathway hemolytic activity (after Mayer) (29). Measured in this way, normal serum had 10.9 CH₅₀ U/ml, serum absorbed with *E. coli* K⁺ had 9.7 CH₅₀ U/ml, serum absorbed with *E. coli* K⁻ had 10.5 CH₅₀ U/ml, and serum absorbed with both organisms had 5.1 CH₅₀ U/ml.

Preparation of IgM and IgG fractions of human serum. Serum was dialyzed against 0.2 M NaCl, 0.1 M Tris-HCl, and 0.02% azide, pH 8.0, and IgM and IgG were separated on a Sephadex G-200 column (Pharmacia Fine Chemicals) as described (29). Appropriate fractions were concentrated 100-fold by pervaporation with a colloid bag apparatus (Schleicher and Schuell, Inc., Keene, N. H.) and tested for IgM and IgG by double immunodiffusion using heavy chain-specific antisera. Fractions free of cross-contamination were combined and designated IgM or IgG fractions; these fractions were adjusted to roughly the same volume as the serum sample from which they were obtained, dialyzed three times against 100-fold volumes of PBS, filtered (0.45 μ m Millipore filter), again demonstrated free of cross-contaminants by double immunodiffusion, and stored at -70°C. Examination of the IgM fraction by immunoelectrophoresis (31) confirmed the absence of IgG. The protein concentrations of the human IgM and IgG fractions were 0.05 and 4.4 mg/ml, respectively (32).

Rabbit anti-*E. coli* K⁺ IgM and IgG. New Zealand white rabbits injected intravenously at 4-d intervals for 12 d with increasing amounts of an *E. coli* K⁺ vaccine prepared as described (33) yielded antiserum with a high titer of both IgM and IgG antibody. IgM and IgG fractions were separated by Sephadex G-200 chromatography, demonstrated free of cross-contamination, and stored as described above for human serum. The protein concentrations of the rabbit IgM and IgG fractions were 0.8 and 6.1 mg/ml, respectively. 1 ml of a 1% suspension of *E. coli* K⁺ in PBS was agglutinated by 25 μ l of IgM or 75 μ l of IgG.

Protein A absorption of rabbit IgM. The rabbit IgM fraction was absorbed with protein A to assure complete removal of IgG by diluting 350 μ l of the IgM fraction 1:9 with PBS and absorbing it twice at 4°C for 1 h with 0.1 ml of gel containing 58 mg of dry protein A-Sepharose CL-4B (Pharmacia Fine Chemicals). This amount of gel was in excess of that required to remove all the IgG from 350 μ l of a 1:9 dilution of the rabbit IgG fraction.

Fluorescence microscopy of complement-coated bacteria. 3×10^6 mid-log phase bacteria were incubated for 15 min at 37°C with 600 μ l of 25% normal or absorbed serum in PBS, washed twice with PBS, incubated for 10 min at 37°C with a mixture containing 20 μ l of rhodamine-conjugated goat anti-human C3 IgG and 80 μ l PBS, washed again, resuspended in 100 μ l of PBS, and examined with a Zeiss Fluorescent Photomicroscope III (Carl Zeiss, Inc., New York). IgM- and IgG-mediated complement fixation to the surface of the *E. coli* K⁺ was determined similarly, except in the initial step 1.5×10^6 bacteria were incubated with 0–60 μ l of human IgM or IgG fraction in a total volume of 300 μ l of 10% absorbed serum.

Attachment of bacteria to phagocytes with Con A. *E. coli* were attached to mouse macrophages with Con A by washing the macrophages three times with PBS (4°C), incubating them with 125 μ g Con A/ml PBS for 30 min at 4°C, again washing them three times with PBS (4°C), and incubating them for 45 min at 4°C with 1 ml of a 2% suspension of encapsulated *E. coli* 09:K29:H⁻ (*E. coli* K⁺) or a 1% suspension of unencapsulated *E. coli* 09:K9⁻:H12 for 45 min at 4°C. *E. coli* were attached to PMN with Con A by incubating a 1% suspension of *E. coli* K⁺ or *E. coli* 09:K9⁻:H12 with subagglutinating amounts of Con A (250 μ g/ml PBS for *E. coli* K⁺ and 8 μ g/ml for *E. coli*

09:K9:H12) for 30 min at 4°C, washing and resuspending them in PBS (4°C), and incubating 1 ml of a 2% suspension of *E. coli* K⁺ or a 1% suspension of *E. coli* 09:K9:H12 with PMN monolayer cultures for 10 min at 37°C; PMN monolayers were prepared as described in Methods for PMN phagocytosis assay I and washed three times in PBS. At the end of the incubations, the monolayers were washed with PBS to remove unbound bacteria.

PMN killing assay. In all killing assays, reactants (total volume 900 μ l) were added to plastic tubes in an ice water bath; the tubes were then gassed with 5% CO₂–95% air so that the pH was 7.4 as judged by the color of the phenol red indicator dye, capped, sealed with Parafilm (American Can Co., Greenwich, Conn.), and incubated at 37°C on a gyratory shaker at 240 rpm. In most experiments, human immunoglobulin, autologous serum (normal, absorbed, or heat inactivated), 5×10^6 PMN in 500 μ l HBSS, or the various controls for these reactants, and 0.5 or 1×10^6 bacteria in 100 μ l of PBS, were added in that order. In experiments involving rabbit immunoglobulin, the immunoglobulin and bacteria were added first and incubated at room temperature for 10 min before other reactants were added as above. A separate tube was used for each time point; thus tubes were not opened until the end of the incubation. At that time, the tubes were placed in an ice water bath to stop the reaction, and the contents were sonicated under sterile conditions with an amount of energy sufficient to lyse leukocytes completely (as measured by phase-contrast microscopy) but not to reduce bacterial CFU. To determine the number of CFU, the contents of each tube were serially diluted in PBS (4°C), spread onto 100 \times 15-mm TSB agar plates using a glass spreader and a rotating wheel (Rota-Plate, Fisher Scientific Co., Pittsburgh, Pa.), incubated overnight at 37°C, and colonies counted manually with a colony counter (New Brunswick Scientific Co., Inc., Edison, N. J.).

Monocyte killing assay. The assay was identical to the above except that mononuclear cells rather than PMN were added to the reaction mixture. On the basis of the differential cell count, 2.5×10^6 monocytes were incubated with 5×10^5 *E. coli* K⁺ (monocyte:bacteria ratio of 5:1).

PMN phagocytosis assay I: PMN in monolayer. PMN (1.25×10^5 in 75 μ l HBSS) were allowed to attach to 13-mm Diam glass coverslips in 16-mm Diam culture wells (Costar Data Packaging, Cambridge, Mass.) for 15 min at 37°C under 5% CO₂–95% air atmosphere after which the various serum preparations and 6.25×10^5 bacteria in a total volume of 300 μ l were added to each well. The wells were gassed with a 5% CO₂–95% air mixture, the entire 24-well unit sealed with Parafilm, and the unit incubated for 20 min at 37°C on a gyratory shaker at 140 rpm. Each coverslip was then washed with ice-cold PBS, fixed in 2.5% glutaraldehyde in PD, mounted on a glass slide, and examined by phase-contrast microscopy; over 100 PMN in random fields were examined, and the percentage of PMN with vacuoles containing bacteria determined.

PMN phagocytosis assay II: PMN in suspension. This assay, which allowed accurate counts of ingested bacteria, was set up exactly as the PMN killing assay involving rabbit immunoglobulin except that a total of 10^7 *E. coli* K⁺ were incubated with 2.5×10^6 PMN (bacteria:PMN ratio of 4:1) for 30 min; afterward, 100- μ l samples from each tube were cyto-centrifuged onto glass slides. The cells were fixed and stained with Diff-Quik Fixative and Solutions (Harleco, American Hospital Supply Corp., Gibbstown, N. J.) and the percentage of PMN with cell-associated bacteria and the number of bacteria per ingesting PMN determined microscopically. As 94% of PMN with cell-associated bacteria had at least one clearly intracellular bacterium by the criteria that the bac-

terium was in a vacuole and/or partially digested and at least 79% of all cell-associated bacteria were clearly intracellular by these criteria, all cell-associated bacteria are tabulated as phagocytosed bacteria (see Results).

Monocyte phagocytosis assay. This assay was identical to PMN phagocytosis assay II except that mononuclear cells rather than PMN were added to the reaction mixture. On the basis of the differential cell count, 10^6 monocytes were incubated with 10^7 *E. coli* K⁺ (bacteria:monocyte ratio of 10:1).

RESULTS

The influence of the capsule on ingestion of *E. coli* attached to phagocytes with Con A and the role of antibody and complement in ingestion of encapsulated and unencapsulated strains. To examine the influence of the capsule on the attachment and ingestion stages of phagocytosis, we first asked whether ingestion automatically follows attachment of *E. coli* to the surface of phagocytic leukocytes or whether antibody and complement are additionally required. At the same time, we asked whether the presence of a capsule influences the fate of bacteria once they are attached. To answer these questions, we required a method of attaching *E. coli* to phagocytes without antibody and complement. For this purpose, we used Con A, as described in Methods; over 98% of macrophages and PMN had adherent bacteria, usually 10–20 bacteria/PMN and 20–50/macrophage.

Encapsulated *E. coli* 09:K29:H[–] (*E. coli* K⁺) bound to the surfaces of resident or thioglycollate-elicited mouse peritoneal macrophages or human PMN with Con A were not ingested on incubation of the bacteria-leukocyte complexes in PBS or minimal essential medium without glucose at 37°C for 1–3 h; glucose-free medium was used because glucose is a competitive inhibitor of Con A and promotes the release of bacteria from the leukocyte surface. In fact, these bacteria multiplied on the surface of the phagocytes. After 1–3 h incubation, these *E. coli* were eluted from the phagocyte surface with 100 mM α MM, a competitive inhibitor of Con A (Fig. 1). In contrast, unencapsulated *E. coli* 09:K9:H12 bound to the phagocytes with Con A were ingested by >95% of the phagocytes on incubation in PBS or minimal essential medium without glucose at 37°C for 1–3 h.

Other experiments confirmed that Con A does not promote phagocytosis of encapsulated *E. coli*. We observed no ingestion when we treated *E. coli*-macrophage complexes (formed as described in Methods or by coating the bacteria with a subagglutinating dose of Con A and incubating them with macrophages for 30 min at 4°C) with excess Con A (125–250 μ g/ml) before (45 min at 4°C) or during the 37°C incubation.

Encapsulated *E. coli* were ingested by >95% of macrophages when 5% rabbit anti-*E. coli* K⁺ antiserum or 5% goat anti-Con A antiserum was added to the

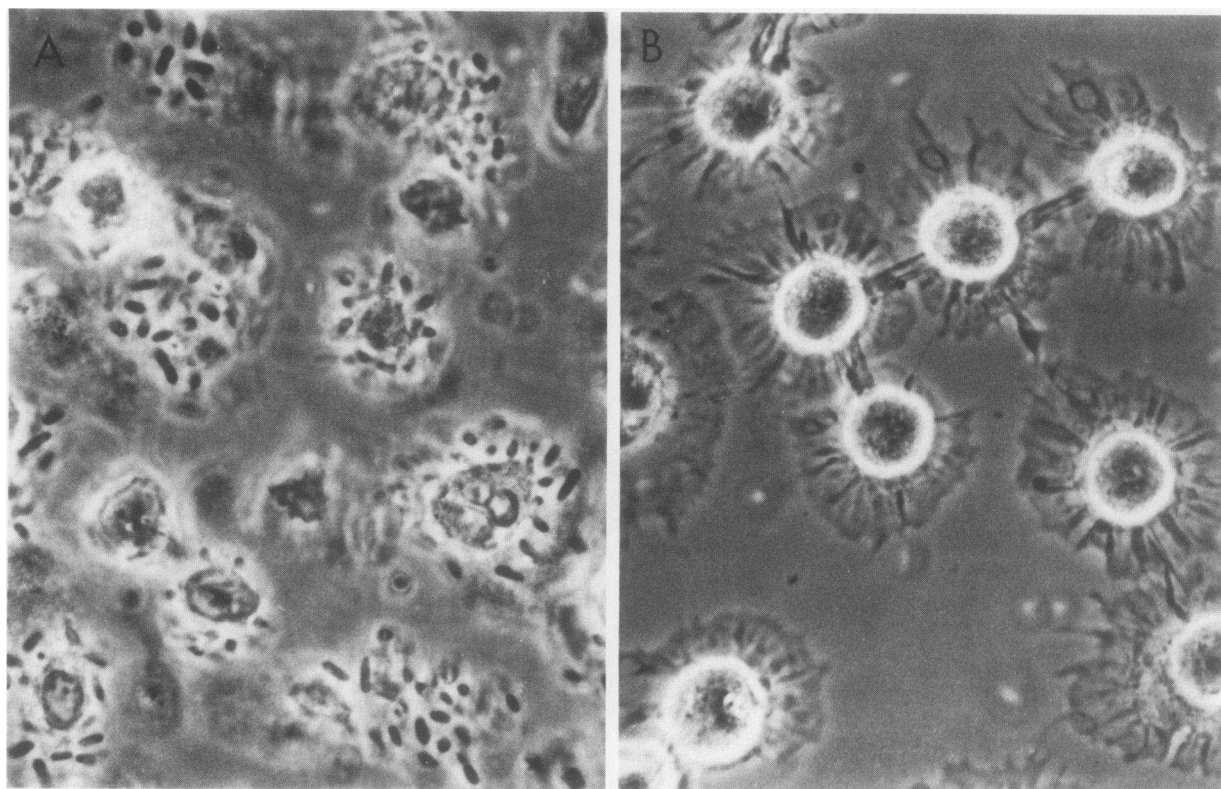


FIGURE 1 PMN do not phagocytose encapsulated *E. coli* attached to the PMN surface with Con A. *E. coli* K⁺—PMN complexes were incubated in PBS at 37°C for 1 h and examined by phase-contrast microscopy before (A) and after (B) treatment with 100 mM α -methyl-D-mannopyranoside. $\times 1,350$.

media containing the complexes of resident or thioglycollate-elicited macrophages and Con A-coated *E. coli*. However, 10–20% fresh mouse serum from non-immune mice (a source of complement but not antibody) did not induce ingestion of these bacteria.

In control experiments without Con A, encapsulated *E. coli* were not attached or ingested when incubated with resident or thioglycollate-elicited macrophages at 37°C for 1 h in the presence or absence of 10% fresh mouse serum. In contrast, unencapsulated *E. coli* 09:K9⁻:H12 were attached and ingested in medium containing fresh mouse serum, but not in serum-free medium.

We derived three conclusions from these experiments: (a) attachment of unencapsulated *E. coli* to the surface of a phagocyte is sufficient to induce ingestion of the unencapsulated bacteria; (b) attachment of encapsulated *E. coli* is insufficient to induce ingestion of these bacteria. Serum ligands, in this instance antibodies to bacterial surface antigens or to Con A on the bacterial surface, are required to promote phagocytosis; (c) in the absence of antibody, fresh mouse serum (a source of complement) promotes attachment and subsequently ingestion of unencapsulated *E. coli*, but is

unable to promote either attachment or ingestion of encapsulated *E. coli*.

The experiments with fresh mouse serum strongly suggested that unencapsulated but not encapsulated *E. coli* fix complement in the absence of antibody and consequently are phagocytosed by phagocytes in the absence of antibody. To test this hypothesis, we studied phagocytosis and killing of an encapsulated *E. coli* (*E. coli* K⁺) and an unencapsulated mutant (*E. coli* K⁻) of the same strain by human PMN and monocytes.

Killing of encapsulated and unencapsulated *E. coli* by human PMN in the presence of normal human serum. To determine the optimal ratio of PMN:bacteria for in vitro killing, we incubated *E. coli* K⁺ and K⁻ with 5×10^6 PMN in 10% autologous normal human serum at 37°C for 1 h so that the ratio of PMN:bacteria ranged from 0.3:1 to 37.5:1. Killing occurred only in the presence of PMN. A 2-log reduction in CFU was obtained independent of the ratio of PMN:bacteria and of the status of encapsulation.

To study the kinetics of killing, we incubated 5×10^5 *E. coli* K⁺ or K⁻ with 5×10^6 PMN (PMN:bacteria ratio of 10:1) in 10% normal serum at 37°C for periods ranging from 0 to 120 min. Bacteria were killed only in

the presence of both PMN and serum (Fig. 2). Maximal killing was achieved by 60 min of incubation, and most or all of this occurred in the first 30 min. The rate of killing of encapsulated *E. coli* was similar to that of unencapsulated *E. coli*.

To study the effect of the concentration of normal serum on PMN killing, we incubated 5×10^5 *E. coli* K⁺ or K⁻ with 5×10^6 PMN at 37°C for 1 h in medium containing 0-20% normal serum. With low serum concentrations (0.1 and 1%), significant (1 log) killing of the *E. coli* K⁻ strain occurred but there was no killing of the *E. coli* K⁺ strain. With higher concentrations of serum (5, 10, and 20%), significant killing (2-2.5 logs) of both *E. coli* K⁺ and K⁻ strains occurred. Maximal killing of both strains occurred with 10 and 20% serum.

Roles of antibody and complement in the killing of encapsulated and unencapsulated *E. coli* by PMN. To examine the role of complement in the absence of antibody in the killing of *E. coli* by PMN, we absorbed normal human serum with *E. coli* K⁺ and K⁻. To grossly compensate for the 31% reduction in classic and 52% reduction in alternative complement pathway activities that occurred with absorption of the serum, we increased the concentration of absorbed serum from 10 to 15% in some samples. Heat-inactivated normal human serum served as a source of antibody. Several lines of evidence, described in later sections of this paper, confirmed that the human serum used in these studies contained antibodies directed against *E. coli* K⁺.

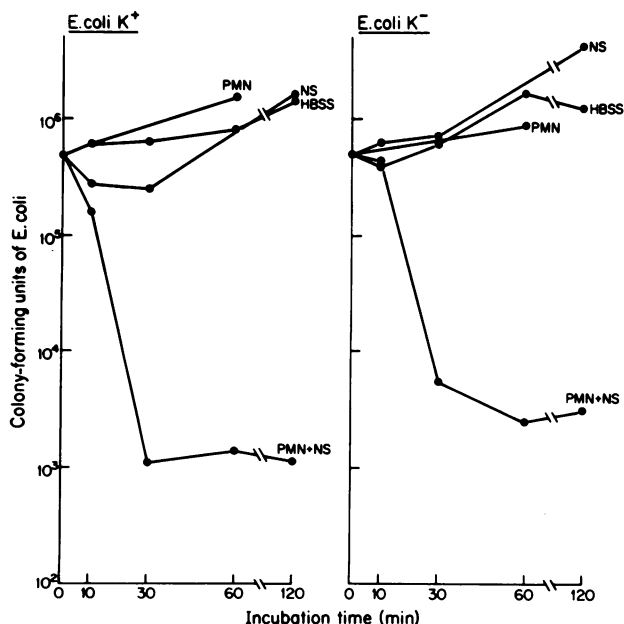


FIGURE 2 Rate of killing of *E. coli* K⁺ and K⁻ by PMN. PMN (5×10^6) were incubated with 5×10^5 *E. coli* K⁺ (left) or *E. coli* K⁻ (right) in 10% normal serum (NS) at 37°C for periods ranging from 0 to 120 min; CFU of *E. coli* were determined initially and at the end of each incubation period.

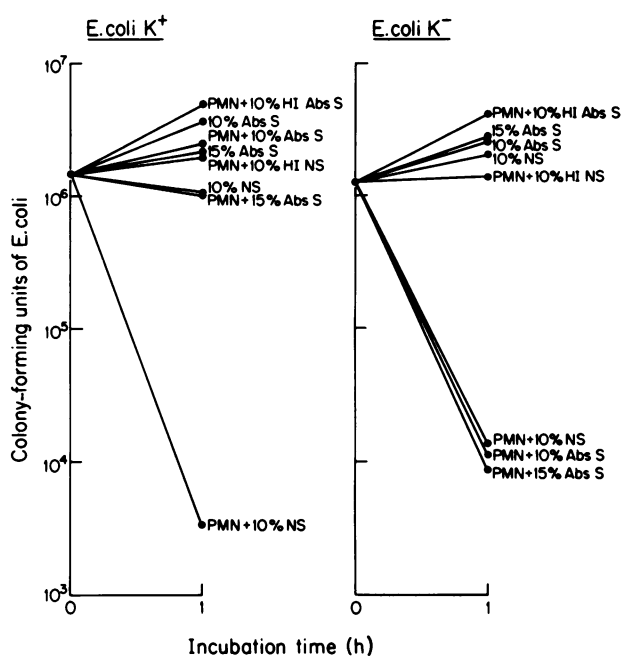


FIGURE 3 PMN killing of *E. coli* K⁺ and K⁻ in the presence of absorbed (Abs S), heat-inactivated (HI), or normal serum (NS). PMN (5×10^6) were incubated with 10^6 *E. coli* K⁺ (left) or *E. coli* K⁻ (right) at 37°C for 1 h in the presence of the serum preparations indicated. CFU of *E. coli* were determined initially and at the end of the incubation.

PMN were unable to kill *E. coli* K⁺ or K⁻ in the presence of heat-inactivated serum (Fig. 3). In the presence of absorbed serum (as a complement source), PMN readily killed *E. coli* K⁻ (2 logs) but were unable to kill *E. coli* K⁺. However, PMN killed *E. coli* K⁺ in the presence of normal serum. We conducted these experiments with a PMN:bacteria ratio of 5:1. We obtained similar results with a PMN:bacteria ratio of 50:1 (5×10^6 PMN: 10^5 bacteria). Thus, the inability of PMN to kill *E. coli* K⁺ in the presence of absorbed serum was not a consequence of an inadequate ratio of PMN:bacteria.

We derived two important conclusions from the results of these experiments: (a) complement is required for the efficient killing of *E. coli* K⁺ and K⁻ by PMN; (b) in the presence of complement, antibody is required to promote killing of encapsulated but not unencapsulated *E. coli* by PMN.

In other experiments, we determined that adequate levels of both antibody and complement are required to achieve maximal killing of *E. coli* K⁺. With the antibody level held constant, PMN did not kill *E. coli* K⁺ significantly with 0.1-1% absorbed serum but killed them maximally (3 logs) with 5-10% absorbed serum (Fig. 4A). Conversely, with the complement level held constant, PMN did not kill *E. coli* K⁺ with 0-0.1% heat-inactivated serum, but killed them significantly (1 log)

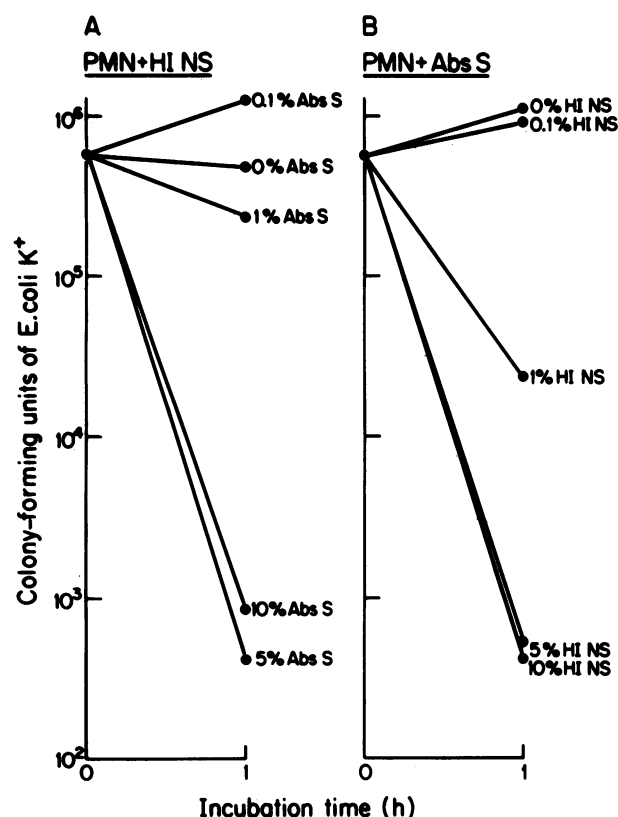


FIGURE 4 Concentration of heat-inactivated normal serum (antibody source) and of absorbed serum (complement source) required for PMN killing of encapsulated *E. coli*. PMN (5×10^6) were incubated with 5×10^5 *E. coli* K⁺ at 37°C for 1 h with a constant amount of heat-inactivated normal serum (5% HI NS) and varying amounts of absorbed serum (Abs S) (A) or a constant amount of absorbed serum (5% Abs S) and varying amounts of heat-inactivated normal serum (B); CFU were determined initially and at the end of the incubation.

with 1% and maximally (3 logs) with 5% heat-inactivated serum (Fig. 4B).

Control experiments showed that in the absence of PMN neither *E. coli* K⁺ nor K⁻ were killed, even when a 50% concentration of normal serum was used. Thus it seemed likely that the differences in serum requirements for PMN killing of *E. coli* K⁺ and K⁻ reflected differences in requirements for phagocytosis.

Roles of antibody and complement in the phagocytosis of encapsulated and unencapsulated *E. coli* by PMN. In our phagocytosis studies, we increased the ratio of bacteria:PMN to 5:1 to facilitate counting and improve the accuracy of the assay.

Without complement (no serum or heat-inactivated serum), PMN did not ingest either *E. coli* K⁺ or K⁻ (Table I). With complement and antibody (normal serum), PMN ingested both *E. coli* K⁺ and K⁻. With complement alone (absorbed serum at concentrations of 10 or 15%), PMN ingested *E. coli* K⁻ as avidly as

TABLE I
PMN Phagocytosis of *E. coli* K⁺ and K⁻ in the Presence of Absorbed, Heat-inactivated, or Normal Serum

Serum preparation	Percentage PMN ingesting	
	<i>E. coli</i> K ⁺	<i>E. coli</i> K ⁻
None	0	0
Normal serum, 10%	83	66
Heat-inactivated normal serum, 10%	0	4
Absorbed serum, 10%	0	65
Heat-inactivated absorbed serum, 10%	0	1
Absorbed serum, 15%	0	67

PMN (1.25×10^5) in a monolayer on coverslips were incubated with 6.25×10^5 *E. coli* K⁺ or K⁻ for 20 min at 37°C in the presence of the serum preparation indicated. At the end of the incubation, the coverslips were washed, fixed, examined by phase-contrast microscopy, and the percentage of PMN with intracellular bacteria determined.

with antibody and complement, but PMN did not ingest *E. coli* K⁺.

Thus, the requirements for PMN phagocytosis of *E. coli* K⁺ and K⁻ precisely mirrored the requirements for PMN killing of these bacteria: antibody and complement were required for phagocytosis of encapsulated *E. coli*, whereas complement alone sufficed for phagocytosis of unencapsulated *E. coli*. The simplest explanation for the differences in requirements between the two organisms was that antibody was required for fixation of complement onto the encapsulated but not the unencapsulated *E. coli*.

Antibody is required to fix complement to encapsulated but not to unencapsulated *E. coli*. To establish the requirements for complement binding to *E. coli* K⁺ or K⁻, we incubated these bacteria with absorbed serum (complement) or whole normal serum (complement and antibody) and then with rhodamine-conjugated goat antihuman C3 IgG and examined them by fluorescence microscopy. After incubation in normal serum (antibody and complement), both *E. coli* K⁺ and K⁻ bound complement as evidenced by very bright fluorescence of the bacteria (Fig. 5). With complement alone, the encapsulated bacteria exhibited no fluorescence but the unencapsulated bacteria fluoresced brightly. Neither type of bacteria exhibited fluorescence when incubated with heat-inactivated normal or heat-inactivated absorbed serum.

Thus, antibody is required for binding complement to encapsulated but not to unencapsulated *E. coli*. The serum requirements for complement binding are identical to those for phagocytosis and killing by PMN. In other words, unless the bacteria are coated with complement, PMN can neither phagocytose nor kill them efficiently.

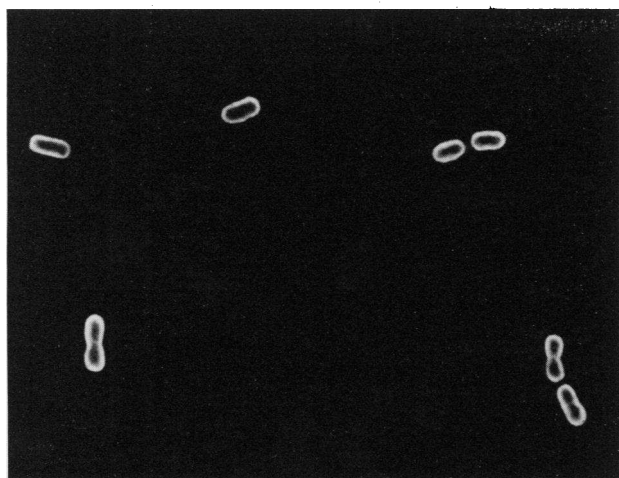


FIGURE 5 Complement fixation to encapsulated *E. coli* in the presence of antibody and complement. *E. coli* K⁺ were incubated in normal serum, washed, incubated in rhodamine-conjugated goat antihuman C3 IgG, and examined by fluorescence microscopy. $\times 1,200$.

Role of the complement and immunoglobulin receptors in killing and phagocytosis of encapsulated E. coli by human PMN. Having determined that phagocytosis and killing of the encapsulated *E. coli* were dependent upon both antibody and complement, we now sought to determine the roles of the C3 and Fc receptors of human PMN in this process. To study C3 receptor function independent of the Fc receptor, we used IgM to fix complement to the bacterial surface. Fc receptors of human PMN do not bind IgM. To study Fc receptor function independent of the C3 receptor, we used IgG in the absence of complement. We obtained human IgM and IgG free of detectable contamination with each other as described in Methods. By fluorescence microscopy, we confirmed that both immunoglobulin preparations promoted the binding of complement to *E. coli* K⁺ in a dose-dependent fashion, i.e., fluorescence appeared and then increased as the amount of immunoglobulin added was increased from 0 to 60 μ l (see Methods).

Both the IgM and IgG fractions efficiently promoted killing of *E. coli* K⁺ by PMN (Fig. 6). When added together, the two immunoglobulins acted synergistically; when 9 μ l of IgG was added to 90 μ l of IgM, killing was enhanced by an additional log over that seen with 90 μ l of IgM alone. Bacterial killing was similarly augmented when 9 μ l of IgM was added to 90 μ l of IgG in the same assay. The amount of each immunoglobulin preparation required for significant bacterial killing (90 and 180 μ l) was roughly equivalent to that present in 10 (90 μ l) and 20% (180 μ l) normal serum, respectively. Control experiments showed that in the absence of complement neither human immunoglobulin fraction promoted killing of *E. coli* K⁺ by PMN.

The capacity of IgM and complement to promote killing of *E. coli* K⁺ by PMN indicates that these bacteria are phagocytosed via PMN complement receptors. This finding was surprising in view of the report of Ehlenberger and Nussenzweig (34) that the C3 receptor of PMN mediates attachment but not phagocytosis of complement-coated sheep RBC, and that Fc receptor function is required for efficient phagocytosis of sheep RBC. To confirm our results, we repeated the killing experiments with high titer rabbit anti-*E. coli* K⁺ IgM and IgG. By double immunodiffusion against goat anti-rabbit IgG, the undiluted rabbit IgM fraction gave no precipitin line, whereas a 1:99 dilution of the IgG fraction yielded a clear precipitin line. To eliminate the possibility that trace amounts of IgG might be contaminating the IgM preparation, we absorbed the rabbit IgM fraction twice with an amount of protein A-Sepharose in excess of that sufficient to remove all opsonic activity from an equal volume of the IgG fraction of this antiserum, as measured by the bacterial killing assay, and all traces of IgG from the same IgG fraction as measured by double immunodiffusion; protein A complexes with >97% of rabbit IgG (35).

In the presence of absorbed serum as a complement source and either rabbit IgG or protein A-absorbed rabbit IgM, PMN killed *E. coli* K⁺ (Table II). With complement and both IgM and IgG, PMN killed significantly more *E. coli* K⁺ than with either immunoglobulin alone. Without complement, PMN killed *E. coli* K⁺ with IgG but not IgM; under these circumstances, killing occurred only at the higher dose of IgG employed (0.04 agglutinating dose). These results confirmed that PMN kill *E. coli* K⁺ in the presence of anti-*E. coli* K⁺ IgM and complement; IgG is not required. With IgG, PMN kill *E. coli* K⁺ more efficiently in the presence of complement than in its absence.

To confirm that PMN killing of bacteria with rabbit

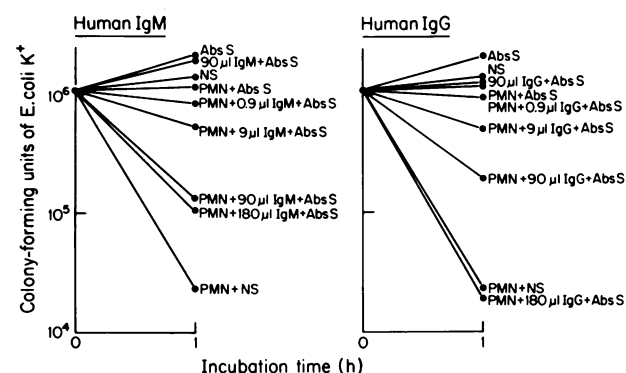


FIGURE 6 PMN killing of encapsulated *E. coli* in the presence of human IgM and IgG. PMN (5×10^6) were incubated with 10^6 *E. coli* K⁺ in the presence of Abs S and varying amounts of human IgM (left) or IgG (right) at 37°C for 1 h; CFU were determined initially and at the end of the incubation.

TABLE II
PMN Killing of Encapsulated *E. coli* in the Presence
of Rabbit IgM and IgG

Antibody preparation	Log change in CFU after incubation with PMN and/or Abs S		
	PMN	Abs S	PMN + Abs S
None		0.4	0
HI rabbit serum (0.20 AD)*	0.2	0.2	-2.4
HI rabbit serum (0.04 AD)	-0.1	0.2	-1.9
IgM (0.04 AD)	0.5	-0.1	-1.3†
IgG (0.04 AD)	-0.8	0.2	-2.5
IgM (0.008 AD)	0.7	0.1	-1.3
IgG (0.008 AD)	0.4	0.1	-1.4
IgM (0.008 AD) plus IgG (0.008 AD)	0.2	0.1	-2.0

PMN (2.5×10^6) and/or Abs S (as a source of complement) were incubated with 4×10^5 *E. coli* K⁺ in the presence of no antibody, heat-inactivated rabbit serum (0.04 and 0.20 AD), protein A absorbed IgM (0.04 and 0.008 AD), IgG (0.04 and 0.008 AD), or IgM plus IgG (0.008 AD of each) at 37°C for 1 h. CFU were determined initially and at the end of the incubation.

* An agglutinating dose (AD) was the minimal concentration of antibody required to agglutinate *E. coli* K⁺ under the conditions described in Methods.

† A -1.3 log change in CFU was also observed in a control sample containing IgM not absorbed with protein A.

immunoglobulin was a consequence of phagocytosis of *E. coli* K⁺, we measured ingestion of these bacteria by PMN using the PMN phagocytosis assay II de-

scribed in Methods. As expected, in the presence of absorbed serum, we found an excellent correlation between the capacity of rabbit IgG or IgM to promote phagocytosis (Table III) and their capacity to promote killing (Table II) of *E. coli* K⁺ by PMN.

Phagocytosis and killing of *E. coli* K⁺ by human blood monocytes. To determine the opsonic requirements for phagocytosis and killing of *E. coli* K⁺ by human blood monocytes, we performed experiments similar to those described above for PMN. As shown in Table IV and Fig. 7, the serum requirements for phagocytosis and killing of *E. coli* K⁺ by monocytes were similar to those observed for PMN. Monocytes phagocytosed and killed *E. coli* K⁺ efficiently only in the presence of complement and antibody (either IgM or IgG).

In obtaining monocytes, we found by chance that if the blood were dextran-sedimented before application to Ficoll-sodium diatrizoate (instead of being applied directly), the mononuclear fraction thus obtained was heavily contaminated with PMN and consisted on the average of 40% monocytes, 36% PMN, and 24% lymphocytes. This allowed a comparison of the phagocytic capabilities of PMN and monocytes obtained in comparable numbers under identical conditions. When we reacted 4×10^6 *E. coli* K⁺ with a mixture containing $\sim 10^6$ monocytes and 10^6 PMN under the same conditions as in Tables III and IV, we found that PMN were strikingly superior to monocytes in phagocytic capability. The phagocytic indices for PMN and monocytes were 620 and 89, respectively, in the presence of IgM and complement, and 370 and 31, respectively,

TABLE III
PMN Phagocytosis of *E. coli* K⁺ in the Presence of Rabbit IgM and IgG

Opsonin(s) added	Percentage of PMN ingesting <i>E. coli</i>	Number of <i>E. coli</i> per PMN ingesting	Phagocytic index*
Normal serum (human)	96	5.6	538
Abs S (human)	2	1.0	2
IgM (0.04 AD)†	0	—	0
IgM (0.04 AD) plus Abs S	92	4.8	441
IgG (0.04 AD)	3	1.3	4
IgG (0.04 AD) plus Abs S	85	2.8	239
HI rabbit serum (0.04 AD)	21	3.3	70
HI rabbit serum (0.04 AD) plus Abs S	99	5.9	585

PMN (2.5×10^6) were incubated with 1×10^7 *E. coli* K⁺ for 30 min at 37°C in the presence of normal human serum (10%) or in the presence of absorbed human serum (10%) and/or 0.04 AD of IgM, IgG, or the heat-inactivated rabbit antiserum from which the immunoglobulins were fractionated. At the end of the incubation, the cells were cytocentrifuged on to glass slides, fixed, stained, examined by bright field microscopy, and the number of PMN ingesting bacteria and the number of such bacteria per ingesting PMN determined as described in detail in Methods.

* Phagocytic index is the percentage of PMN with ingested *E. coli* K⁺ multiplied by the average number of *E. coli* K⁺ ingested per PMN.

† See legend for Table II.

TABLE IV
Monocyte Phagocytosis of *E. coli* K⁺ in the Presence of Rabbit IgM and IgG

Opsonin(s) added	Percentage of monocytes ingesting <i>E. coli</i>	Number <i>E. coli</i> per monocyte ingesting	Phagocytic index
Normal serum (human)	83	4.5	374
Abs S (human)	1	1.0	1
IgM (0.04 AD)*	0	0	0
IgM (0.04 AD) plus Ab S	49	3.2	157
IgG (0.04 AD)	11	2.6	29
IgG (0.04 AD) plus Abs S	53	3.0	160
HI rabbit serum (0.04 AD)	21	2.2	46
HI rabbit serum (0.04 AD) plus Abs S	79	4.9	389

Monocytes (1×10^6) were incubated with 1×10^7 *E. coli* K⁺ under the same conditions as for PMN in Table III, and phagocytosis was assayed in the same way as described for PMN in Table III.

* See legend for Table II.

in the presence of IgG and complement; in these preparations, nearly all the bacteria were in PMN. However, with IgG or heat-inactivated antiserum alone, we found that monocytes and PMN had comparably low phagocytic capacities (indices ≤ 40). This suggested that the complement receptor of the PMN is more efficient than the complement receptor of the monocyte in promoting phagocytosis of complement-coated encapsulated bacteria, but that the Fc receptors of the two phagocytes are comparably efficient.

DISCUSSION

The surfaces of most unencapsulated gram-negative bacteria are rich in lipopolysaccharide. In the absence of antibody, it is presumably the interaction of this surface with complement components that activates the complement pathway and leads to the deposition of complement onto the bacterial surface; lipopolysaccharide has been shown capable of fixing complement without antibody (36). Our experiments indicate that the *E. coli* capsule blocks complement fixation to the bacterial surface, probably by masking surface components, such as lipopolysaccharide, capable of activating the complement pathway. The capsule itself does not promote complement binding; complement is bound to the surface of encapsulated *E. coli* only when specific IgM or IgG antibody is present. Once complement is bound, encapsulated and unencapsulated *E. coli* are equally phagocytosed and killed by PMN.

Unencapsulated *E. coli*, when attached to phagocytic leukocytes by Con A, do not require either complement or antibody for ingestion; however, under physiologic conditions, i.e., in the absence of plant lectins, they require complement. This suggests that under physiologic conditions, complement and complement receptor activity are required for attachment but not ingestion of unencapsulated *E. coli*. In contrast, encap-

sulated *E. coli*, even when attached to the leukocyte surface with Con A, require serum opsonins such as antibody or complement for ingestion. It follows that under physiologic conditions, complement and/or Fc receptor activity are required for both the attachment and the ingestion stages of phagocytosis of encapsulated *E. coli*. Griffin et al. (37) have come to a similar conclusion in their studies of phagocytosis of IgG and complement-coated RBC by mouse peritoneal macrophages.

Encapsulated *E. coli* coated with IgM and complement are phagocytosed by PMN and monocytes; these

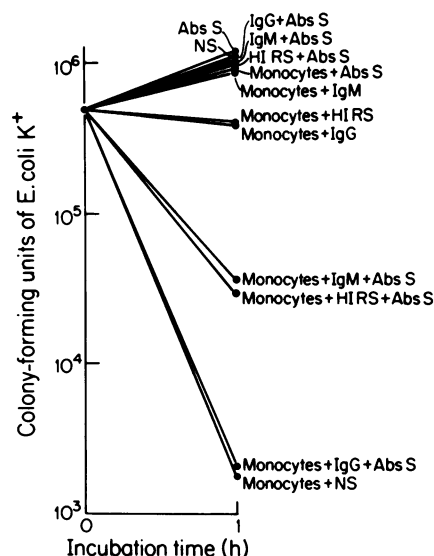


FIGURE 7 Monocyte killing of encapsulated *E. coli* in the presence of rabbit IgM and IgG. Monocytes (2.5×10^6) were incubated with 5×10^5 *E. coli* K⁺ in the presence of Abs S and rabbit IgM or IgG at 37°C for 1 hr; CFU were determined initially and at the end of the incubation. HI RS, heat-inactivated rabbit serum.

leukocytes lack receptors for IgM. This indicates that the complement receptors of PMN and monocytes mediate the ingestion of complement-coated encapsulated bacteria. In contrast, Ehlenberger and Nussenzweig have reported that the complement receptors of PMN and monocytes do not promote the ingestion of complement-coated sheep RBC (34). We cannot explain these differences in the response of PMN and monocytes to complement-coated sheep RBC and encapsulated *E. coli*.

The relative roles of complement and antibody in promoting phagocytosis of bacteria coated with these ligands are of more than academic interest. Our results indicate that a host lacking specific IgM or IgG antibacterial antibody might be susceptible to infection with encapsulated but not unencapsulated bacteria. Early in the course of an infection, IgM may be the only antibacterial antibody present. Inasmuch as PMN and monocytes have no IgM receptors, complement would be the only serum ligand available to promote clearance of encapsulated bacteria.

Three points should be taken into account in studying complement binding to bacteria. First, the state of encapsulation of the bacteria is an important determinant of their capacity to bind complement. The amount of capsular material present may vary with different culture conditions. Second, serum must be thoroughly absorbed to remove natural and cross-reacting antibody from "nonimmune" serum; otherwise, a requirement for antibody for complement fixation may be missed. Third, serum must be absorbed with both encapsulated and unencapsulated bacteria to remove all antibacterial antibody. Absorption with encapsulated bacteria alone does not effectively remove antibody to subcapsular surface antigens such as the somatic O antigen. In studies whose details are not included here, we found that serum absorbed with only *E. coli* K⁺ promoted phagocytosis and killing of *E. coli* K⁺. Serum absorbed with both *E. coli* K⁺ and K⁻ was inactive in the absence of added antibody (Figs. 3, 4, 6, and 7; Tables I-IV). These findings suggest that antibodies directed against somatic antigens can react with encapsulated *E. coli* and promote the binding of complement to their surface. The reports of Kaijser et al. (15), and Kaijser and Olling (14) that antibody to the somatic O antigen of encapsulated *E. coli* protected animals against infection with these bacteria are consistent with this view. On the other hand, a large capsule may render subcapsular surface antigens totally inaccessible to antibody, thus preventing antibody to the O antigen from mediating complement fixation; Howard and Glynn (4) reported that anti-OK antibody but not anti-O antibody enhanced the clearance of intravenously injected encapsulated *E. coli*.

Complement consumption by bacteria may not be synonymous with complement binding to the bacteria.

Bacteria release substances such as proteases and endotoxin that cause complement consumption in the fluid phase (38). Similarly, complement fixation to isolated bacterial structures (e.g., cell walls, cell membranes, peptidoglycan) may not be relevant to the in vivo situation where anatomical barriers such as a capsule may limit access of complement components to these structures in the intact bacterium. For these reasons, we studied complement binding to intact bacteria directly by using rhodamine-conjugated anti-human C3 IgG.

The fluorescent antibody assay for complement fixation described in this paper is a simple, rapid, sensitive, reliable, and inexpensive technique for determining whether antibody is required to fix the third component of complement to the surface of any bacterium of interest. To screen a particular bacterium with this assay, one requires only appropriately absorbed serum. Because antibody is required for complement binding and phagocytosis of encapsulated *E. coli*, determining whether or not antibody is required to bind complement to other bacterial species may provide insight into requirements for antibody in host defense against them.

On the basis of our findings, we postulate the following model for the interaction of bacteria (except intracellular parasites) with the host humoral and cellular defense systems. When unencapsulated gram-negative bacteria encounter serum, their lipopolysaccharide-rich outer membrane promotes complement fixation to the bacterial surface. Consequently, the bacteria are either lysed by late-acting complement components or ingested and removed by phagocytic leukocytes. When encapsulated gram-negative bacteria encounter serum, complement is not fixed to the bacterial surface in the absence of specific antibacterial antibody. Consequently, encapsulated bacteria are resistant to complement lysis and phagocytosis. This may account, at least in part, for the enhanced pathogenicity of encapsulated gram-negative bacteria. If specific antibody is available, complement is bound and the encapsulated bacteria are either lysed or phagocytosed.

This model may also apply to gram-positive bacteria. The capsules of gram-positive organisms are also important determinants of virulence; in the case of pneumococcus, the role of anticapsular antibodies in host defense has been recognized for a half century. The outer surface of the unencapsulated gram-positive bacteria, a cell wall containing peptidoglycan, has been shown to promote complement activation (39, 40) and Wilkinson et al. (41) have provided evidence that the capsule of *Staphylococcus aureus* hinders phagocytosis by masking the underlying peptidoglycan layer. Thus, the principles derived from our studies of encapsulated and unencapsulated *E. coli* may be relevant to encapsulated and unencapsulated gram-positive bacteria as well.

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